Modulation of Lon protease activity and aconitase turnover during aging and oxidative stress

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Abstract **We compared Lon protease expression in murine skeletal muscle of young and old, wild-type and *Sod2*$^{-/+}$ heterozygous mice, and studied Lon involvement in the accumulation of damaged (oxidized) proteins. Lon protease protein levels were lower in old and oxidatively challenged animals, and this Lon deficiency was associated with increased levels of carbonylated proteins. We identified one of these proteins as aconitase, and another as an aconitase fragmentation product, which we can also generate in vitro by treating purified aconitase with H_2O_2. These results imply that aging and oxidative stress down-regulate Lon protease expression which, in turn, may be responsible for the accumulation of damaged proteins, such as aconitase, within mitochondria.**

Key words: Lon protease; Aconitase; Protein oxidation; Mitochondria; Aging; *Sod2*

1. Introduction

The physiological functions and the substrate specificity of the Lon protease in mammalian mitochondria are not well understood. In yeast, loss of Lon function results in irregularly shaped mitochondria in which the matrix space becomes filled with electron-dense inclusion bodies thought to be aggregated proteins [1,2]. New data from our laboratory indicate that the Lon protease can selectively degrade oxidatively modified aconitase at a much higher rate than unoxidized control substrate, and may thus play an important role in defending against the accumulation of oxidized proteins in mitochondria [3–5]. Another proposed protein substrate for Lon is the SP-22 mitochondrial protein [6]. This novel protein has analogies with the thiol-specific antioxidant gene (TSA) product from *S. cerevisiae* and has the ability to scavenge reactive oxygen species [7].

Aconitase plays a key function in cellular energy production, and loss of its activity has a major impact on cellular and organismal survival. Oxidative inactivation of aconitase has been associated with decreased lifespan in *Drosophila* [8]. A decline in aconitase activity has also been described in other abnormal situations, such as increased oxidative stress caused by mitochondrial manganese superoxide dismutase (MnSOD) deficiency [9], and in a number of neurodegenerative diseases, including progressive supranuclear palsy [10], Friedreich's ataxia [11], and Huntington's disease [12]. The hypothesis that the Lon protease might have a role in defenses against free radical damage inside mitochondria and that defects in its production can lead to aging and disease [13,14] is in concordance with high-density oligonucleotide array data findings that *lon* mRNA levels decline about four-fold in ageing skeletal murine muscles, and that this phenomenon can be prevented by caloric restriction [15].

In order to study regulation of the Lon protease during aging and oxidative stress, we have chosen a murine model: mitochondrial *Sod2*$^{-/+}$ (heterozygous B6-*Sod2* < *tm1* < *Cje* knockout) mice [9]. Because of their deficiency in antioxidant (MnSOD) defenses [9], these mice suffer a considerable amount of oxygen radical damage [16] and have altered mitochondrial function [17]. Both mitochondrial superoxide dismutase gene (*Sod2*) knockout null mutants are lethal and display a whole spectrum of disorders characteristic of pathological ageing, such as neurodegeneration and myocardial injury, at an early age [18,19]. The heterozygous *Sod2*$^{-/+}$ mice are viable, but they exhibit a 50% decrease in MnSOD activity and altered mitochondrial function [20], with accumulation of 8-hydroxydeoxyguanosine in the mt-DNA, and increased carbonyl groups in mitochondrial proteins [9]. In older animals, an increased incidence of lymphoma, hydro-nephrosis and pituitary adenoma has been reported [16].

2. Materials and methods

2.1. Materials

All chemicals and reagents were obtained from Sigma unless otherwise specified. Purified aconitase was exposed to various concentrations of hydrogen peroxide at 37°C and pH 7.4 as previously described [21], except that a shorter incubation time (30 min) was used. Both the oxidized aconitase and the controls were extensively dialyzed before use for Western blotting.

2.2. Animals

The line used in this study was B6-*Sod2* < *tm1* < *Cje* [9,16,20]. The *Sod2*$^{-/+}$ female mice and their wild-type, age-matched, controls were fed ad libitum and maintained under barrier conditions on a 12-h dark/light cycle. Mice aged 3 to 6 months (young animals) and 27 months (old animals) were killed by cervical dislocation, and the hind limb muscles were immediately excised and frozen.

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Abbreviations: MnSOD, manganese superoxide dismutase; *Sod2*, mitochondrial superoxide dismutase gene; *Sod2*$^{-/+}$, heterozygous B6-*Sod2* < *tm1* < *Cje* knockout mice; *Sod2*$^{+/+}$, wild-type littermate control mice; SDS, sodium dodecyl sulfate; SDC, sodium deoxycholate

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2.3. Isolation of muscle proteins

The total hind leg muscle mass (1.5 g) was cut in small pieces, homogenized with a glass homogenizer in the presence of 5 ml lysis buffer [50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM PMSF, 1mM EDTA, 5 μg/ml Aprotinin, 5 μg/ml Leupeptin, 1% Triton X-100, 1% sodium deoxycholate (SDC), 0.1% sodium dodecyl sulfate (SDS)], and centrifuged for 30 min in an Eppendorf tube at maximum speed (14 000*g*); all these steps were performed at 4°C. The supernatant was saved as a whole tissue lysate, and stored at −80°C for future study.

2.4. Preparation of antibodies and Western blot analysis

The anti-Lon polyclonal antibody was raised in rabbits against a synthetic peptide corresponding to amino acids 593–609 of the intact human Lon protease, and it cross-reacts strongly with murine Lon. In muscle extracts the anti-Lon polyclonal antibody recognized mostly one protein band with an estimated molecular size of approximately 100 kDa, which corresponds to the molecular size previously described for both the human and bovine Lon protease [22–24]. The anti-aconitase polyclonal antibody was targeted to residues 767–780 of the C-terminus of human mitochondrial aconitase and cross-reacts with both murine and porcine mitochondrial aconitase. The anti-aconitase antibody specifically recognized an 83-kDa band, the size of intact mitochondrial aconitase. When oxidized aconitase (Sigma) was used, a number of fragmentation and aggregation products were also detected. ProSci produced both antibodies to our specifications.

For Western blot detection we always used pooled samples from six different animals from each group. We loaded 40 μg of protein extract for Lon protease detection, and 20 μg of either protein extract or purified oxidized aconitase (Sigma) for aconitase identification per lane. After separation in 10–20% gradients gels, the proteins were transferred onto BioRad PVDF membranes. The membranes were incubated with the anti-Lon antibody at a 1:1000 dilution, or with the anti-aconitase antibody at a 1:2000 dilution as indicated in the figure legends. The secondary antibody in both cases was a goat anti-rabbit IgG (Jackson ImmunoResearch Lab), and we used a 1:20 000 dilution. Next, antigens were detected by chemiluminesce with Amersham's ECL substrates. On all the Western blots, the membranes were stripped and reprobed for cytochrome *c* (as a loading control for mitochondrial proteins) and tubulin (total loading control).

2.5. Protein oxidation measurement

To detect oxidized proteins, we used the Oxyblot™ kit from Intergen Company, based on derivatization of carbonyl groups in the presence of 2,4-dinitrophenylhydrazine (DNPH), using a primary antibody against dinitrophenylhydrazone (DNP) moieties for detection. We loaded 40 μg protein in each lane. The blots were stripped and reprobed with the anti-aconitase antibody to confirm the aconitase identity.

3. Results and discussion

3.1. Changes in expression of the Lon protease in murine muscle with aging and decreased MnSOD activity

Lon protease levels were lower in skeletal muscles of old wild-type littermate control mice ($Sod2^{+/+}$) and both young and old heterozygous $Sod2^{-/+}$ mice (Fig. 1) when compared with the young $Sod2^{+/+}$. When we quantified our Western blots (using IP lab gel software), the optical density of the Lon protease band was four times lower in the old $Sod2^{+/+}$, two times lower in the young $Sod2^{-/+}$ group, and six times lower in the old $Sod^{-/+}$ group, in comparison with the young $Sod2^{+/+}$ animals. These results suggest that a major Lon protease down-regulation is caused both by aging and by a reduction in (MnSOD) antioxidant defense systems, and that these two effects are additive.

The Lon protease is a major factor in maintaining mitochondrial integrity and continued mitochondrial biogenesis [1,2,25]. We have previously shown that the Lon protease degrades oxidized aconitase in cell culture systems, and that cells with low levels of Lon protease accumulate oxidized (inactive) aconitase and therefore have decreased total aconitase activity [4,5]. Thus decreased Lon levels can lead to deficient energy production and incomplete removal of oxidized proteins, phenomena frequently described in skeletal muscles of old or diseased individuals.

3.2. Mitochondrial aconitase levels are not changed by aging or oxidative stress

Our Western blots showed no significant differences in the levels of aconitase between the four experimental groups (Fig. 2). However, in addition to the traditionally described 83-kDa aconitase band, we were able to detect another aconitase fragment of 40 kDa, as well as a number of less distinctive bands, which form a smear in the old and $Sod2^{-/+}$ mice. The 40-kDa fragment decreased with age and oxidative stress, whereas other fragmentation products with molecular weights between 40 and 83 kDa increased with age and MnSOD deficiency.

As mentioned in the Section 1, a decline in aconitase activity has been previously measured in our $Sod2^{-/+}$ mouse model [9]. Since we detected almost equal levels of aconitase protein in the present studies, the previously described loss of activity [9] must not have been caused by a decreased amount of aconitase protein. No difference in aconitase levels in the liver of $Sod2^{+/+}$ and $Sod2^{-/+}$ was also previously described [9]. We propose that partial replacement of normal aconitase with inactive (oxidatively) damaged enzyme is the major cause of diminished intracellular aconitase activity in the $Sod2^{-/+}$ mouse.

3.3. Age and MnSOD down-regulation increase the levels of carbonylated proteins

The total level of carbonylation increased both with aging and with oxidative stress, to a 36% increase in old $Sod2^{+/+}$ mice, an 80% increase in young $Sod2^{-/+}$ and a 250% increase

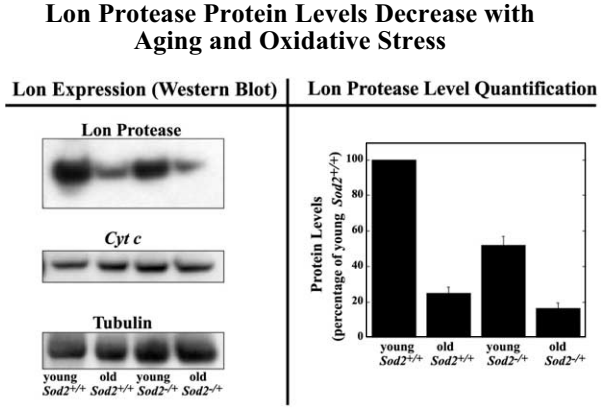

Fig. 1. Mitochondrial Lon protease protein levels decrease with aging and oxidative stress. Protein extracts were prepared from hind leg muscles of young and old $Sod2^{+/+}$ and $Sod2^{-/+}$ mice. Samples from six individual animals from each experimental group were pooled together, as described in Section 2. The levels of Lon protease protein, cytochrome *c* (cyt *c*) and tubulin were determined by Western blot, as described in Section 2. Each gel lane contained a combined sample (40 μg) from six different animals. The panel on the right shows a summary of means and standard errors of Lon protease levels from three different experiments, visualized by Western blotting, and quantified by densitometry (IP Lab), in which results are reported as percentages of Lon expression in young $Sod2^{+/+}$ animals.

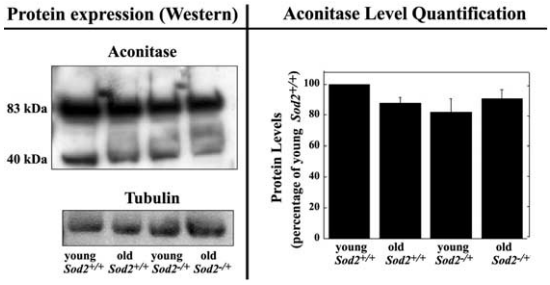

Fig. 2. Mitochondrial aconitase protein levels remain unchanged with aging and oxidative stress. The content of aconitase in total muscle protein samples from the four groups of animals was estimated by Western blotting, as described in Section 2, in three independent experiments. The blots were quantified by densitometry using IP Lab software, and the results (means and standard errors) are expressed as percentages of total aconitase content in young, wild-type animals. Tubulin was used as a loading control. Each lane contained 20 μg protein coming from six individual mice per each experimental group.

in the old $Sod2^{-/+}$ mice, when compared with young $Sod2^{+/+}$ mice (Fig. 3A,B).

Using Western blot analysis of different carbonylated proteins, we were able to identify at least seven distinct bands (depending on the exposure time) corresponding to molecular weights of 205 kDa, 130 kDa, 83 kDa, 70 kDa, 60 kDa, 50 kDa and 40 kDa (Fig. 3A). Each of these seven proteins had a different pattern of expression in the four groups of animals (Fig. 3B), with the higher molecular weight bands (205 kDa and 130 kDa) being detectable only in the $Sod2^{-/+}$ animals, suggesting that these bands could be formed by aggregation of heavily damaged (oxidized) proteins. By stripping and reprobing the gels of Fig. 3 with the anti-aconitase antibody (Fig. 4A), we confirmed that the 83-kDa band was aconitase, but the 40-kDa band also cross-reacted with the primary antibody, allowing us to identify it as the aconitase fragmentation product that we described in Fig. 2. In young $Sod2^{+/+}$ mice the 40-kDa carbonylated fragment was approximately 50% of

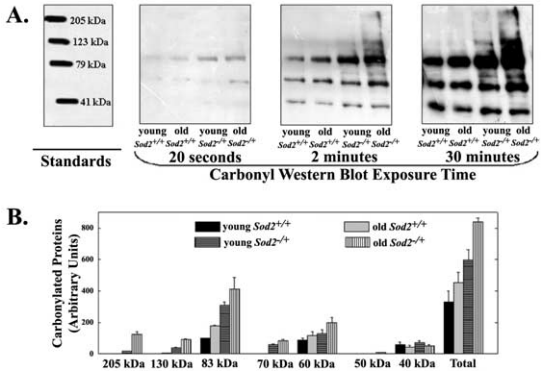

Fig. 3. Carbonylated muscle proteins in young and old, normal and $Sod2^{-/+}$ mice. Oxidized (carbonylated) muscle proteins (40 μg/lane) were identified by derivatization of carbonyl groups in the presence of DNPH, and Western blotting using anti-DNP antibodies (for details see Section 2). A: Varying exposure times allowed the differentiation of seven carbonylated proteins, the most prominent band corresponding to a molecular weight of 83 kDa. B: Densitometric quantification of three independent experiments. The levels of the most distinctive bands were calculated as percentages (means and standard errors) of the 83-kDa band in the young wild-type animals.

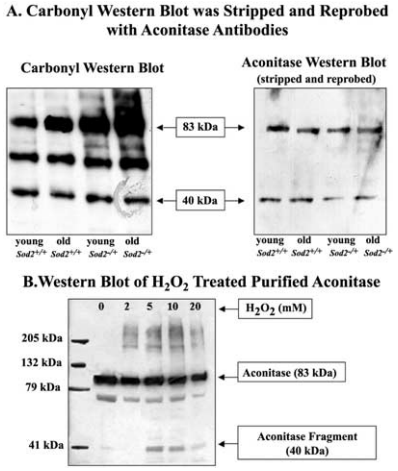

Fig. 4. Carbonylated aconitase and aconitase fragmentation product identification. A: After carbonylated protein detection (see Fig. 3), the membranes were chemically stripped and reprobed with primary anti-aconitase antibodies. Only two different products, with molecular weights of 83 kDa and 40 kDa, were identified in repeated experiments. B: Purified aconitase (0.33 mg/ml) was incubated with H_2O_2 at the concentrations indicated for 30 min at 37°, as described in Section 2. Residual peroxide was then removed by dialysis, and aconitase (both oxidized and control) was used for Western blot detection (primary anti-aconitase antibody). The blot shown in B was repeated several times with similar results.

the level of carbonylated aconitase (83 kDa; Fig. 4A). Interestingly, the absolute levels of the 40-kDa band did not change with age or *Sod2* deficiency, but the relative amounts, when compared with the level of carbonylated aconitase in the same sample, actually decreased (to 12% of the level of carbonylated aconitase in old $Sod2^{-/+}$ mice). The identity of the 40-kDa protein fragment was also verified by in vitro H_2O_2 treatment of purified aconitase (Sigma; Fig. 4B). At lower levels of peroxide treatment this fragment was not seen, but it appeared at H_2O_2 concentrations above 2 mM. In agreement with our observations in murine muscles, this fragment was not detected at very high oxidant treatment (20 mM H_2O_2), probably because of further fragmentation or loss of antigenicity.

We measured very significant differences in the levels of oxidized aconitase (the 83-kDa band) in the carbonyl blots of the various groups of animals (Fig. 3A,B). Carbonylated aconitase accumulated in old $Sod2^{+/+}$ mice (almost twice as much as in the young animals) and in both the young $Sod2^{-/+}$ and old $Sod2^{-/+}$ (three and four times more, respectively) mice when compared to the levels in the young $Sod2^{+/+}$ mice. This accumulation of oxidized aconitase is consistent with previously published reports, suggesting that aconitase is a selective target for oxidation [8]. However, aconitase was not the only protein that was modified and it always accounted for less than 50% of the total carbonyl content in our quantifications (Fig. 3B).

In conclusion, we propose that a decline in Lon protease expression is possibly a major significant factor in the accumulation of oxidized mitochondrial aconitase in old and oxidatively challenged animals. Since accumulation of damaged proteins can lead to mitochondrial dysfunction, we also propose that diminished Lon activity plays a significant role in the development of mitochondrial pathology during aging. In addition, a decrease in mitochondrial biogenesis, the other

proposed effect of Lon deficiency in mammalian cells [1,25], would render muscle fibers unable to produce enough new mitochondria to replace the old and damaged ones, and would lead to impaired energy metabolism in muscle tissues.

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